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# Intrinsic Temperature Sensitivity of Influenza C Virus Hemagglutinin-Esterase-Fusion Protein

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**Influenza C virus replicates more efficiently at 33°C than at 37°C. To determine whether hemagglutinin-esterase-fusion protein (HEF), a surface glycoprotein of influenza C virus, is a restricting factor for this temperature sensitivity, we analyzed the biological and biochemical properties of HEF at 33°C and 37°C. We found that HEF exhibits intrinsic temperature sensitivities for surface expression and fusion activity.**

The optimum temperature for the replication of influenza C viruses, which cause mainly upper respiratory tract diseases (7, 8), is around 32 to 33°C (12, 19). Studies have demonstrated that influenza C viral RNA polymerase has higher activity at 33°C than at 37°C (10, 11). To obtain further insights into the intrinsic temperature sensitivities of influenza C viruses, we investigated hemagglutinin-esterase-fusion protein (HEF), an influenza C virus glycoprotein (14). HEF is the counterpart of both hemagglutinin (HA) and neuraminidase (NA) in influenza A and B viruses and has three biological properties: receptor binding, receptor destruction, and membrane fusion activity (5, 13).

To examine whether influenza C virus grows better at a lower temperature due to its HEF functions, African green monkey kidney-derived CV-1 cells were infected with C/Ann Arbor/1/50 (C/AA50) virus at a multiplicity of infection (MOI) of 10 and cultured at either 33°C or 37°C. At 48 h postinfection, infected cells were treated with tosylsulfonyl phenylalanyl chloromethyl ketone (TPCK)-trypsin and exposed to fusion buffer (phosphate-buffered saline with 10 mM morpholineethanesulfonic acid and 10 mM HEPES; adjusted to pH 5.0). After a 4-h incubation, polykaryon formation was examined under a microscope. We found that polykaryons were formed in 75% of the cells at 33°C compared with 35% of the cells at 37°C (Fig. 1A). We then analyzed HEF expression levels on infected cells by flow cytometry with the anti-HEF monoclonal antibody (MAb) clone J14 (17). The HEF cell surface expression level at 33°C was twice that at 37°C (Fig. 1B). These results suggest that the better growth of influenza C virus at the lower temperature is associated with HEF functions.

To determine whether intrinsic HEF biologic properties contribute to the temperature sensitivity of this virus, we wanted to rule out the influence of other viral proteins on HEF expression and/or function. To this end, we expressed C/AA50 HEF by using a protein expression plasmid, pME18S/HEF-AA (9). HEF expression from this plasmid was controlled by an RNA polymerase II-driven simian virus 40 early promoter. To test the effect of temperature on HEF expression, COS-1 cells, derivatives of CV-1 cells with a high transfection efficiency, were transfected with the plasmid and cultured at 37°C for 24 h, followed by either further incubation at 37°C or a temperature shift to 33°C. At 24 and 48 h posttransfection (hpt), transfected cells were labeled with [$^{35}$S]methionine for 20 min. Cell lysates were immunoprecipitated with anti-HEF MAb D37 (18) and analyzed by means of sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE). The HEF expression levels at both temperatures were comparable (Fig. 2A), indicating that the effect of temperature on HEF expression from the HEF expression plasmid was limited.

Next, to test the effect of temperature on HEF cell surface expression, COS-1 cells were transfected with the HEF expression plasmid, and at 24 hpt or later, the culture temperature was shifted to 33°C (Fig. 2B). At 48 hpt, the HEF expression level on the transfected cells was examined by using flow cytometry. The HEF expression levels on the surface of the transfected cells increased as the culture time at 33°C increased; the mean fluorescence intensity of HEF expressed on the cells that were kept at 37°C was half

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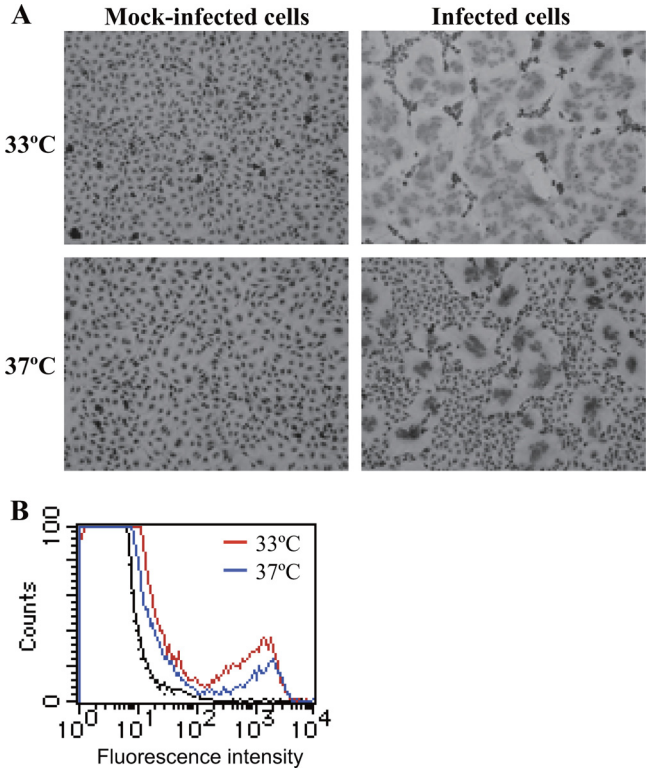


**FIG 1** Polykaryon formation and HEF cell surface expression levels at different temperatures in influenza C virus-infected cells. CV-1 cells were mock infected (as controls) or infected with C/AA50 virus at an MOI of 10 and cultured at either 33°C or 37°C for 48 h. (A) Infected cells were treated with 50 μg/ml TPCK-trypsin, exposed to fusion buffer (pH 5.0), and further incubated at 33°C or 37°C in neutral pH medium for 4 h. Cells were then stained with Giemsa solution. (B) Infected cells were incubated with anti-HEF MAb J14 on ice for 60 min, stained with a fluorescein isothiocyanate-conjugated secondary antibody on ice for 30 min, and subjected to flow cytometric analysis for HEF expression. Results with mock-infected cells are shown by the black line.

that of the HEF expressed on the cells cultured at 33°C for 24 h (Fig. 2C).

We next investigated the temperature sensitivity of HEF membrane fusion activity. HEF-expressing COS-1 cells prepared as described above were treated with TPCK-trypsin and exposed to fusion buffer. After a 4-h incubation, polykaryon formation was examined. HEF-mediated polykaryon formation was observed only with cells incubated at 33°C for over 12 h (Fig. 2D). Although these results suggest that HEF membrane fusion activity is higher at the lower temperature than that at the higher temperature, the fusion efficiency may have been influenced by the level of HEF cell surface expression (Fig. 2C). Indeed, membrane fusion mediated by influenza A virus HA is correlated with the level of HA cell surface expression (3).

To further investigate the temperature sensitivity of HEF membrane fusion activity, we assessed the HEF membrane fusion activity by using a more sensitive assay. For influenza A viruses, HA-mediated cell-erythrocyte fusion requires fewer HA molecules per cell than does polykaryon formation (4). We therefore tested the temperature sensitivity of HEF-mediated cell-erythrocyte fusion. The membrane fusion process is divided into three steps: membrane contact, hemifusion (lipid mixing), and aqueous pore formation (content mixing) (15). To assess the latter two steps separately, mouse erythrocytes were labeled with lipophilic octadecyl rhodamine B chloride (R18; Molecular Probes) and aqueous calcein-AM (Dojindo, Japan) to monitor hemifusion and pore formation, respectively, as described previously (6). COS-1 cells transfected with the HEF expression plasmid were cultured at 37°C for 24 h, followed by either further incubation at 37°C or a temperature shift to 33°C. At 48 hpt, transfected cells were treated with TPCK-trypsin, incubated with R18- or calcein-labeled erythrocytes on ice for 10 min, and then exposed to the fusion buffer (pH 5.0). The transfer of R18 from the mouse erythrocytes to the COS-1 cells was comparable at both 33°C and 37°C (Fig. 2E). In contrast, the calcein transfer at 33°C was more efficient (i.e., it was detected in 71% of cells) than that at 37°C (detected in only 41% of cells), as shown by the presence of the remaining erythrocytes. These results suggested that the restriction of HEF-mediated membrane fusion at the higher temperature was due to the lower efficiency of fusion pore formation.

Proper oligomerization of influenza virus glycoproteins that are synthesized in the endoplasmic reticulum is critical for their transport to the plasma membrane via the Golgi apparatus (2). To determine the mechanism by which temperature influences events involving HEF cell surface expression, we examined HEF oligomerization by using sucrose velocity sedimentation, as previously described (16). HEF expression plasmid-transfected COS-1 cells were cultured at 37°C for 24 h, followed by either further incubation at 37°C or a temperature shift to 33°C. At 48 hpt, transfected cells were labeled with [$^{35}$S]methionine for 20 min and further cultured for 2 h at either 33°C or 37°C. Cell lysates were then subjected to sucrose velocity sedimentation on 5%-to-20% sucrose gradients. Fractions were immunoprecipitated with rabbit antiserum against C/AA50 virions (20) and analyzed by SDS-PAGE. Bovine serum albumin (BSA; 67 kDa) and human IgG (156 kDa) served as sucrose gradient standards and were run on parallel gradients. Monomeric (80 kDa) and trimeric forms of HEF were detected with peaks in fractions 8 to 11 and fractions 3 to 5, respectively (Fig. 3). Two independent experiments revealed that the ratio of trimer to monomer at 33°C was higher than that at 37°C, and a large amount of aggregates was observed in fraction 1 at 37°C, indicating that HEF is oligomerized at 33°C more efficiently than at 37°C. These results also suggest that inefficient oligomerization at the higher temperature is responsible for the reduced level of HEF cell surface expression at that temperature (Fig. 2C).

Here, we demonstrated that the influenza C virus glycoprotein HEF is expressed on the cell surface (Fig. 2C) and induces membrane fusion (Fig. 2D and E) more efficiently at 33°C than at 37°C. HEF oligomerization was also more efficient at 33°C than at 37°C (Fig. 3). These results indicate that HEF has an intrinsic temperature sensitivity that is regulated by its oligomerization status. Our findings suggest that the more efficient replication of influenza C viruses at lower temperatures, such as those in the upper respiratory tract, is conferred by greater biological activity of not only the viral RNA polymerase but also of HEF.

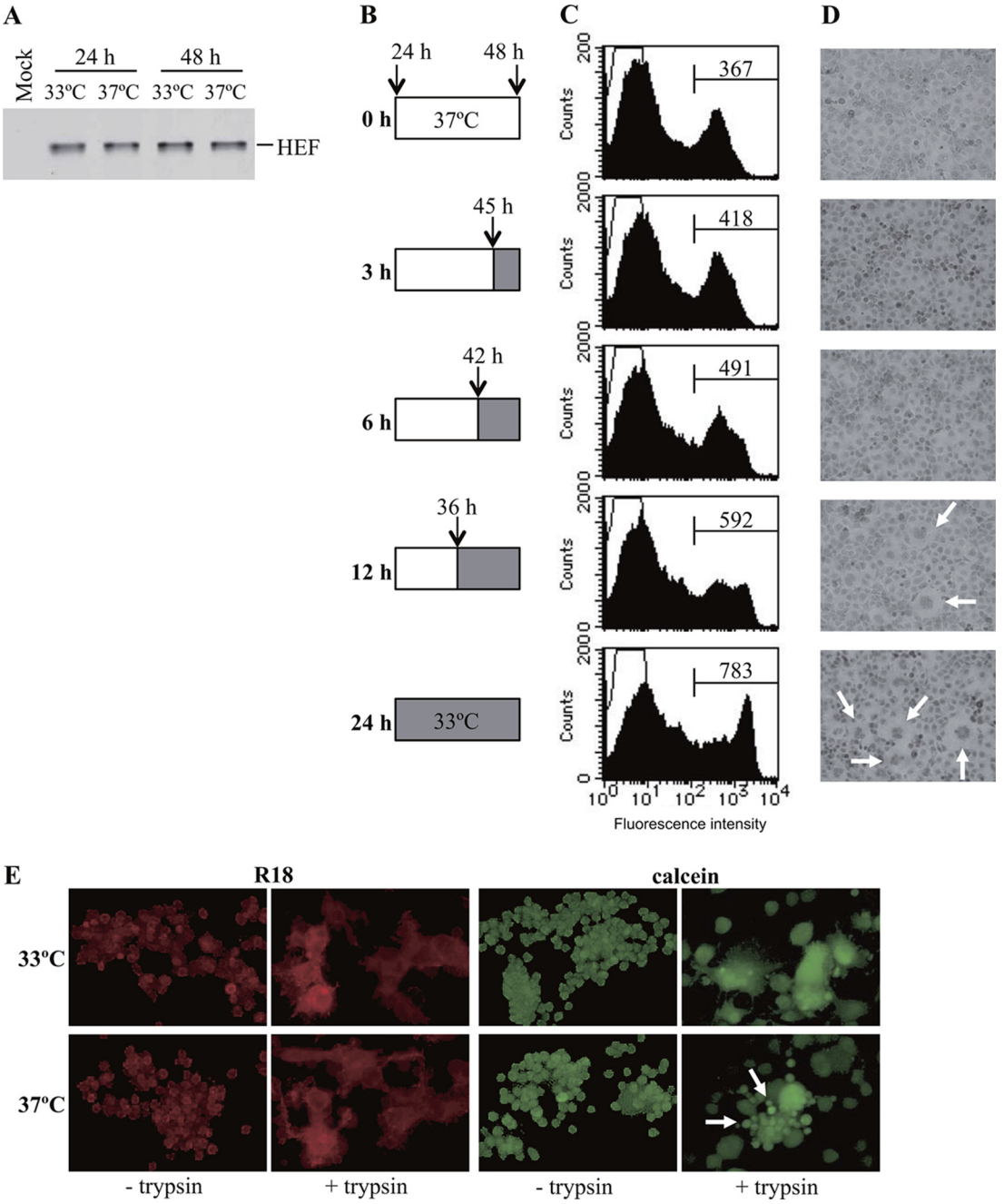


**FIG 2** Effects of temperature on HEF cell surface expression levels and membrane fusion activity. (A) COS-1 cells transfected with 1.0 μg of pME18S/HEF-AA were cultured at 37°C for 24 h, followed by further incubation at either 33°C or 37°C for 24 h. At 24 and 48 hpt, transfected cells were labeled with [$^{35}$S]methionine for 20 min. Cell lysates were immunoprecipitated with anti-HEF MAb D37 and analyzed by SDS-PAGE. Images were captured by using a charge-coupled-device camera and quantified by using ImageJ software (1) (the results shown are representative data from three independent experiments). (B to D) COS-1 cells transfected with 1.0 μg of pME18S/HEF-AA were cultured at 37°C. (B) At the indicated time points, the culture temperature was shifted to 33°C. (C) At 48 hpt, transfected cells were incubated with anti-HEF MAb J14 on ice for 60 min, stained with a fluorescein isothiocyanate-conjugated secondary antibody on ice for 30 min, and subjected to flow cytometric analysis for HEF expression. The black line indicates results for mock-transfected cells. Mean fluorescence intensity values are shown. (D) At 48 hpt, transfected cells were treated with 50 μg/ml TPCK-trypsin, exposed to fusion buffer (pH 5.0), and further incubated at 33°C or 37°C in neutral pH medium for 4 h. Cells were then stained with Giemsa solution. White arrows indicate polykaryons. (E) COS-1 cells transfected with 1.0 μg of pME18S/HEF-AA were cultured at 37°C for 24 h, followed by further incubation at either 33°C or 37°C for 24 h. At 48 hpt, transfected cells were left untreated or were treated with 50 μg/ml TPCK-trypsin and then incubated with R18- or calcein-prelabeled mouse erythrocytes on ice for 10 min. After removing unbound erythrocytes, cells were exposed to fusion buffer (pH 5.0). Fluorescent signals were detected by using a fluorescence microscope. White arrows indicate the remaining erythrocytes.

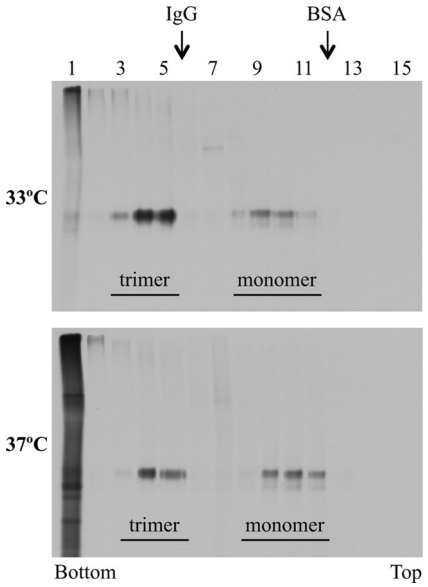


**FIG 3** Effect of temperature on HEF oligomerization. COS-1 cells transfected with 1.0 μg of pME18S/HEF-AA were cultured at 37°C for 24 h, followed by further incubation at either 33°C or 37°C for 24 h. At 48 hpt, transfected cells were labeled with [$^{35}$S]methionine for 20 min and further cultured for 2 h at either 33°C or 37°C. Cell lysates were then subjected to sucrose velocity sedimentation on 5%-to-20% sucrose gradients. Fractions were immunoprecipitated with rabbit antiserum against C/AA50 virions and analyzed by SDS-PAGE. BSA and human IgG served as sucrose gradient standards. The peak fractions for the standards, run on parallel gradients, are indicated with arrowheads. The results shown are representative data from two independent experiments.

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